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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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☐ *Additional inventors are being named on the____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

INTERFERON-*BETA* POLYMER CONJUGATES

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 38 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 1 ☐ Other (specify)

☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

SIGNATURE

Date 03/01/2004

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Docket Number: 213.1204

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This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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Invention: INTERFERON-BETA POLYMER CONJUGATES

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INTERFERON-*BETA* POLYMER CONJUGATES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is directed to *beta* interferon -polymer conjugates. In particular, the invention is directed to polymer conjugates of interferon *beta* 1b and substantially non-antigenic polymers such as PEG.

Description of the Related Art

Many proteins or polypeptides are known that hold great promise for use in treating a wide variety of diseases or disorders. Unfortunately, protein therapeutics suffer from a number of drawbacks, including poor solubility in water and body fluids, rapid clearance from the bloodstream after administration, and the potential to elicit an immune response from the treated person or animal. One proposed solution to addressing these drawbacks is to conjugate such proteins or polypeptides to substantially non-antigenic polymers in order to improve circulating life, water solubility and/or to reduce antigenicity. For example, some of the initial concepts of coupling peptides or polypeptides to polyethylene glycol (PEG) and similar water-soluble polymers are disclosed in U.S. Pat. No. 4,179,337, the disclosure of which is incorporated herein by reference.

Interferons, also referred to herein as IFNs, are one class of therapeutic proteins that will benefit from improved circulating life, water solubility and/or reduced antigenicity. Interferons are relatively small polypeptide proteins which are secreted by most animal cells in response to exposure to a variety of inducers. Because of their antiviral, antiproliferative and immunomodulatory properties, interferons are of great interest as therapeutic agents. They exert their cellular activities by binding to specific membrane receptors on the cell surface. Once bound to the cell membrane, interferons initiate a complex sequence of intracellular events. *In vitro* studies demonstrated that these include the induction of certain enzymes, suppression of cell proliferation,

immunomodulating activities such as enhancement of the phagocytic activity of macrophages and augmentation of the specific cytotoxicity of lymphocytes for target cells, and inhibition of virus replication in virus-infected cells. Thus, interferon proteins are functionally defined, and a wide variety of natural and synthetic or recombinant interferons are known. There are three major types of human IFNs. These are:

Leukocyte IFN or IFN-*alpha*, a Type 1 IFN produced *in vivo* by leukocytes.

Fibroblast IFN or IFN-*beta*, a Type 1 IFN produced *in vivo* by fibroblasts.

Immune IFN or IFN-*gamma*, a Type 2 IFN produced *in vivo* by the immune system.

IFN-*beta* is of particular interest for the treatment of a number of diseases or disorders, and especially in the treatment of multiple sclerosis or MS. Natural human IFN-*beta* is a 166 amino acid glycoprotein, and the encoding gene has been sequenced by Taniguchi, *et. al.*, 1980, Gene 10: 11-15, and R. Derynck, *et al.*, *supra*. Natural IFN-*beta* has three cysteine (cys) residues, located at amino acid positions 17, 31 and 141, respectively. In addition, numerous recombinant variants of IFN-*beta* are known.

Three recombinant IFN-*beta* products are licensed in Europe and the U.S. for treatment of MS. These are interferon *beta*-1a ("IFN-*beta*-1a") or Avonex® (Biogen, Inc., Cambridge, Massachusettes), another IFN-*beta*-1a product marketed as Rebif® (Ares-Serono, Norwood, Massachusettes) and Ser_{17} interferon-*beta*-1b ("IFN-*beta*-$1b_{Ser17}$") or Betaseron® (Berlex, Richmond, California).

IFN *beta*-1a is produced in mammalian cells, e.g., Chinese Hamster Ovary ("CHO") cells using the natural human gene sequence, and the produced protein is glycosylated. *See*, for example, U.S. Patent Nos. 5,795,779, 5,376,567 and4,966,843, incorporated by reference herein. IFN *beta*-1b Ser_{17} differs structurally from IFN-*beta*1a (Avonex® and Rebif®) because it is produced in *Escherichia coli* ("*E. coli*") using a modified human gene sequence having an engineered cysteine-to-serine substitution at amino acid position 17, so that the protein is non-glycosylated. *See*, e.g., U.S. Patent Nos. 4,588,585 and 4,737,462, the disclosures of which are incorporated by reference herein.

Both Rebif® and Avonex® are stated by their package inserts to have specific activities, by differing methods, of at least 2-3 x 10^8 international units (IU)/mg. The Betaseron® package insert reports a specific activity of approximately 3 x 10^7 IU/mg, indicating a ten-fold difference in potency. While these activities are determined by somewhat different methods, the order of magnitude differences in antiviral and antitumor activities are also reflected in the recommended doses, which are measured in micrograms (60-130 mcg/week) for the Rebif® and Avonex® glycosylated IFN-*beta* 1a products, and from 0.25 milligrams and up for the non-glycosylated Betaseron® IFN-*beta* 1b.

IFN-*beta*, in each of its recombinant formulations, has multiple effects on the immune system, including the ability to inhibit viral replication. IFN-*beta*-1b is described by the manufacturer (Berlex, Richmond, California) as enhancing suppressor T cell activity, reducing proinflammatory cytokine production, down-regulation of antigen presentation, and inhibition of lymphocyte trafficking into the central nervous system. Other sources have reported that IFN-*beta* reduces the production of IFN-gamma by T-lymphocytes. Other beneficial therapeutic effects are also suspected.

However, as with all protein therapeutics, the drug is rapidly cleared from the bloodstream by nonspecific mechanisms, including renal filtration. In addition, patients injected with IFN-*beta* develop anti-IFN-*beta* neutralizing antibodies ("Nabs"). Nabs are a subset of binding antibodies that work to inhibit the normal biological effects of the eliciting antigen, and if elicited by a therapeutic protein, may reduce treatment efficacy. The risk of anti-IFN-*beta* Nab development and subsequent effects on treatment, may preclude early treatment of MS with interferon drugs – a consequence that would significantly curtail the therapeutic promise of these agents. Each of the marketed interferon *beta* drugs is associated with the development of Nabs in clinical trials during treatment of MS. In one two-year study of Betaseron®, nearly half of the treated patients, in both high-and low-dose groups, developed Nabs at some point in the study.

Some further disadvantages of the interferon therapeutics are physical instability i.e. protein aggregation, denaturation and precipitation to name a few and chemical

instability, i.e. deamidation, hyrolysis, disulfide exchange, oxidation etc. Protein aggregation as used herein refers to the formation of dimers, trimers, tetramers, or multimers from monomers which may or may not precipitate in the formulation buffers and conditions of the present invention. The formulation of Ribif, Avonex and Betaseron presently involve the use of HSA which can contribute to viral contamination as well as aggregation of the protein.

Polymer conjugates of IFN-*beta*'s are known. U.S. Pat. Nos. 4,766,106 and 4,917,888, incorporated by reference herein, describe, *inter alia*, amide-linked IFN-*beta* 1b conjugates using mPEG-N-succinimidyl glutarate or mPEG-N-succinimidyl succinate. The patentees disclose that PEGylation of the protein is done using relatively high molar excesses of the activated polymer. Although linkage of the polymer to Lys residues is preferred, N-terminal–polymer linkages as well as those involving Cys, Glu and Asp are also disclosed. See column 8, lines 34-40 of the '888 patent, for example. Published PCT patent application No. WO99/55377 which describes site-selective modification of IFN-*beta* 1a at Cys-17 using a thiol-reactive PEGylating agent, describes shortcomings with the '106 and '888 results, however. Specifically, page 4, lines 5-18 of the PCT application state that although non-specific PEGylation using large molar excesses of activated PEG provided conjugates improved solubility, "a major problem was the reduced level of activity and yield".

Commonly-assigned U.S. Patent No. 5,738,846 discloses preparing various PEG-interferon conjugates. Column 14, line 1 thereof mentions IFN-*beta* as a suitable interferon for conjugation with various forms of activated PEG. Fractionation of the PEGylated product to recover specific species including the mono-PEGylated conjugates is also disclosed.

U.S. Patent No. 5,109,120, incorporated by reference herein, describes methods of making PEG conjugates having an imidoester linker, including generally, IFN-*beta*. U.S. Pat. No. 6,531,122, describes IFN-*beta* variants or muteins different from IFN-*beta* 1b optionally conjugated to polymers such as PEG, including linkage via engineered Cys or Lys residues. Pepinsky, et al. in published U.S. Patent Appl. No. 20030021765 describe IFN-*beta* 1a polymer conjugates, including PEG conjugates and uses thereof. However,

a 20 kDa N-terminal PEGylated IFN-*beta* 1a conjugate failed to provide prolonged effects on a biological marker for IFN-*beta* activity, despite prolonged presence in the serum of test animals (Pepinsky et al., 2001, The Journal of Pharm. and Exper. Ther. 297(3): 1059-1066). In addition, the Pepinsky report noted that a 30 kDa N-terminal PEGylated IFN-*beta* 1a conjugate retained only one-sixth of the activity of the 20 kDa N-terminal PEGylated IFN-*beta* 1a conjugate and a 40 kDa N-terminal PEGylated IFN-*beta* 1a conjugate lost all interferon activity.

Despite the foregoing, it should be noted that the various types of IFN proteins exhibit significant homology differences. For example, IFN-*alpha* and IFN-*beta* exhibit an average homology of only 3% in the domain of the signal sequence and of only 45% in the IFN polypeptide sequence, e.g., as described by Derynck, 1980 Nature, 285: 542-547. In addition, even though there is greater homology among the IFN-*beta*'s, there are nonetheless some significant differences between the two, both in terms of therapeutic use, indications, etc.

In spite of the above-described disclosures, there remains a longstanding and heretofore unsolved need in the art for improved polymer-conjugated IFN-*beta* compositions, particularly those containing IFN-*beta* 1b. There also continues to be a need for improved compositions containing polymer-conjugated IFN-*beta* 1b wherein the polymer has a molecular weight of about 30 kDa (number average), or greater and which are free of human serum albumin (HAS).

SUMMARY OF THE INVENTION

The above-described needs are addressed, and other advantages are provided, by the polymer-conjugated IFN-*beta* compositions described herein. In one aspect of the invention, there is provided an improved biologically-active polymer-interferon conjugate composition. The composition includes an interferon-*beta* 1b conjugated to a polyalkylene oxide (PAO) polymer having a molecular weight of at least about 20 kDa. Preferably, the PAO is a polyethylene glycol (PEG) having a molecular weight of from about 30kDa to about 60 kDa. In one aspect of the invention, the polymer is linked to amino terminal of the IFN, while in other separate and preferred aspects of the invention

the polymer is attached via an epsilon amino group of a Lys of the interferon-*beta* 1b. Depending upon the site of attachment and molecular weight of the polymer selected, retained anti-viral activities for the conjugates will range from at least about 65 percent for the 30 kDa polymer conjugates and at least about 15% for the 40 kDa polymer conjugates. In both cases, the amount of retained activity is significantly greater than that which was expected.

The composition of the present invention incorporates the polymer conjugate described above in the presence of certain buffers and excipients to increase the physical and chemical stability. A further improvement involves providing a HSA free formulation to reduce the risk of viral contamination and protein aggregation.

Other aspects of the invention include methods of making the conjugate compositions or formulations as well as methods of treatment using the same.

As a result of the present invention there are provided improved IFN-*beta* 1b polymer conjugate compositions. The retained anti-viral activity of the conjugates of the present invention is surprising high, especially in view of the fact that the polymer portion thereof is in most aspects of the invention at least about 30 kDa. The prior art, see Pepinsky et al., 2001, The Journal of Pharm. and Exper. Ther. 297(3): 1059-1066, *supra*, which reported that IFN-*beta* 1a, a glycosylated and more potent form of IFN-*beta* as compared to IFN-*beta* 1b, was substantially less active or inactive when the same molecular weight types of PEG were used.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing comparative data discussed in Example 8.

Fig. 2 is a graph showing comparative data discussed in Example 9.

DETAILED DESCRIPTION OF THE INVENTION

Accordingly, the invention provides a composition comprising:

a) an interferon conjugated to a polyalkylene oxide polymer having a molecular weight of at least about 20 kDa; and optionally b) a surfactant; c) an

excipient, and d) a buffer, wherein the pH range of the solution is from about 3.0 to about 11.

In the embodiments herein the compositions have a pH from about 3.0 to about 8.0. In other embodiments, the compositions have a pH from about 3.0 to about 5.0, with a pH of from about 3.0 to about 4.0 being most preferred. The ionic strength of the compositions provided herein has been found to affect the stability i.e. prevent aggregation. Low ionic strength is preferred in low pH buffers while high ionic strength is preferred in high pH buffers. In one embodiment, the ionic strength of a composition on the invention having a pH of from about 3.0 to about 4.0 is lower than 10 mM. In another embodiment, the ionic strength of a composition of the invention having a pH of from about 5.5 to about 7.5 is about 100 to about 150 mM.

The interferon used in the compositions of the invention is preferably interferon-*beta* 1b and more preferably IFN-*beta*-$1b_{Ser17}$.

A. *Beta* Interferons

The term "interferon-beta" or "IFN-*beta*" as used herein refers to IFN-*beta* isolated from natural sources and/or produced by recombinant DNA technology as described in the art, having sequence homology with, and the functionality, including bioactivity, of native IFN-*beta*. The term "interferon-beta 1b" or "IFN-*beta* 1b" as used herein refers to a mutein of IFN-*beta* having residue Cys_{17} replaced by residue Ser_{17}, and expressed in a nonglycosylated form, with the N-terminal amino acid, Methionine, post-translationally removed, and represented herein as SEQ ID NO:1.

As noted in more detail, *supra*, the *beta* interferon (IFN-*beta*) portion of the polymer conjugate can be prepared or obtained from a variety of sources, including recombinant techniques such as those using synthetic genes expressed in suitable eukaryotic or prokaryotic host cells, e.g., *see* U.S. Patent No. 5,814,485, incorporated by reference herein. In addition, the IFN-*beta* can also be a mammalian source extract such as human, ruminant or bovine IFN-*beta*. One particularly preferred IFN-*beta* is IFN-

beta-1b$_{Ser17}$, a recombinantly-made product available from Berlex, (Richmond, California), as described by U.S. Patent No. 4,737,462, incorporated by reference.

The IFN-*beta* proteins employed to produce the inventive conjugates were either obtained commercially, e.g., IFN-*beta* 1b was obtained from Berlex, Inc. (Richmond, California) or produced and isolated as exemplified hereinbelow. Native human IFN-*beta* is optionally employed, although it is preferred to use an IFN-*beta* mutein optimized for production and solubilization in a prokaryotic host. One preferred prokaryotic host cell is *Escherichia coli.*

Many muteins of the native human or animal IFN-*beta* are known and contemplated to be employed in the practice of the invention. Preferred muteins are described in greater detail by U.S. Patent Nos. 4,588,585, 4,959,314;,4,737,462 and 4,450,103, incorporated by reference herein. In brief, as noted above, a preferred mutein is one wherein the Cys_{17} residue of native human IFN-*beta* is replaced by serine, threonine, glycine, alanine, valine, leucine, isoleucine, histidine, tyrosine, phenylalanine, tryptophan or methionine. Most preferred is the non-glycosylated Ser_{17} mutein of IFN-*beta*, also referred to herein as IFN-*beta* 1b.

Numerous methods of expressing and isolating IFN-*beta* proteins from prokaryotic host systems, and vectors suitable for expression by prokaryotic host cells, are known. For example, much of the IFN-*beta* employed in the examples provided hereinbelow was produced by the following method. A synthetic gene encoding an IFN-*beta*, e.g., IFN-*beta* 1b, was synthesized, following codon optimization for bacterial expression.

Other methods and reagents for IFN-*beta* production and purification are described, for example, by U.S. Patent Nos. 6,107,057, 5,866,362, 5,814,485, 5,523,215, 5,248,769, 4,961,969, 4,894,334, 4,894,330, 4,748,234, 4,656,132, all incorporated by reference herein, as well as by other references too numerous to mention.

Methods of expressing and isolating IFN-*beta* proteins, and vectors suitable for expression by eukaryotic host cells, such as Chinese Hamster Ovary ("CHO") cells, are

described in detail, e.g., by U.S. Patent Nos. 4,966,843, 5,376,567, and 5,795,779, incorporated by reference herein.

B. Non-Antigenic Polymers

The polymeric portion of the conjugate useful in the compositions of the invention can be linear and is preferably selected from the group consisting of:

A- O-$(CH_2CH_2O)_x$-
A-O-$(CH_2CH_2O)_x$-$CH_2C(O)$-O-,
A-O-$(CH_2CH_2O)_x$-CH_2CH_2 NR_7-,
A-O-$(CH_2CH_2O)_x$-CH_2CH_2 SH,
-O-$C(O)CH_2$-O-$(CH_2CH_2O)_x$-$CH_2C(O)$-O-,
-$NR_7CH_2CH_2$-O-$(CH_2CH_2O)_x$-CH_2CH_2 NR_7-,
-$SHCH_2CH_2$-O-$(CH_2CH_2O)_x$-CH_2CH_2 SH-,

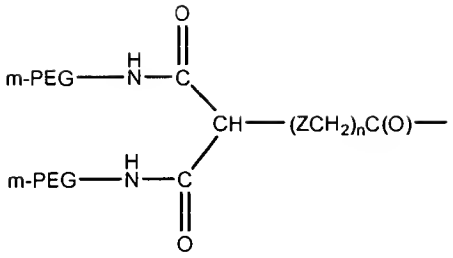
wherein

A is a capping group;

R_7 is selected from hydrogen, C_{1-6} alkyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{3-8} substituted cycloalkyls, aryls, substituted aryls, aralkyls, C_{1-6} alkenyls, C_{3-12} branched alkenyls, C_{1-6} alkynyls, C_{3-12} branched alkynyls, C_{1-6} heteroalkyls, substituted C_{1-6} hetero-alkyls, C_{1-6} alkoxyalkyl, phenoxyalkyl and C_{1-6} heteroalkoxys, and

x is the degree of polymerization. The variable x is preferably a positive integer selected so that the molecular weight of the polymer is within the ranges disclosed herein, i.e. 20- 60 kDa, as being preferred.

Alternatively the polymeric portion of the conjugate useful in the compositions of the invention can be branched and is preferably selected from the group consisting of:



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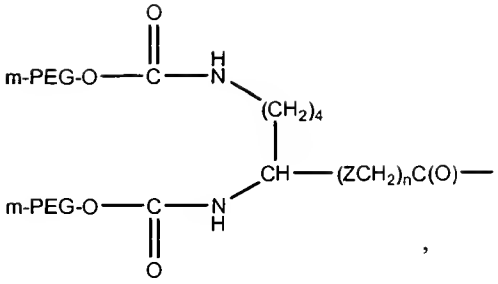

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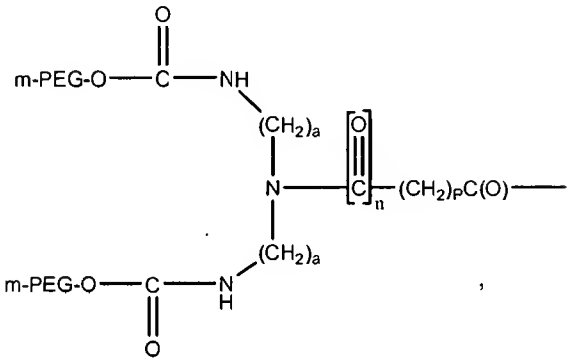

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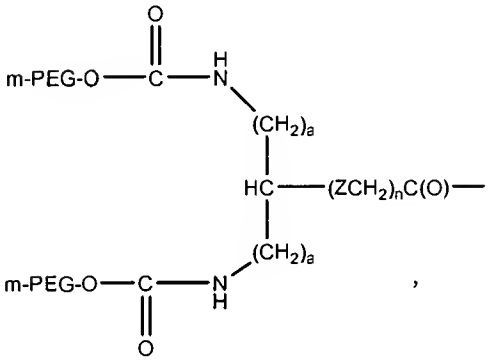

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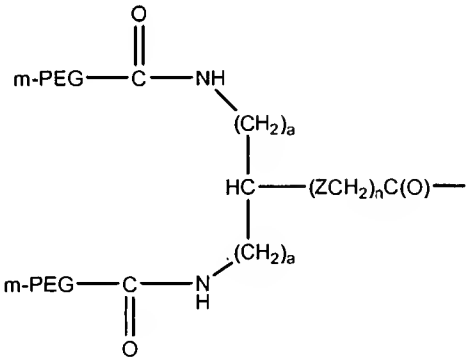


,

wherein:

(a) is an integer of from about 1 to about 5;

Z is O, NR_8, S, SO or SO_2; where R_8 is H, C_{1-8} alkyl, C_{1-8} branched alkyl, C_{1-8} substituted alkyl, aryl or aralkyl;

(n) is 0 or 1;

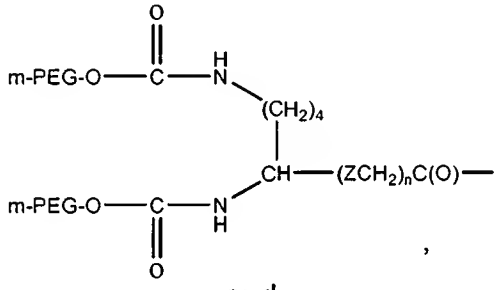
(p) is a positive integer, preferably from about 1 to about 6, and

m-PEG is CH3-O-$(CH_2CH_2O)_x$- .

Preferably, the capping group A is selected from the group consisting of OH, CO_2H, NH_2, SH, and C_{1-6} alkyl moieties.

More preferably, interferon –beta 1b is conjugated to a polyalkylene oxide polymer selected from the group selected from:

A- O-$(CH_2CH_2O)_x$-
A-O-$(CH_2CH_2O)_x$-$CH_2C(O)$-O-,
A-O-$(CH_2CH_2O)_x$-CH_2CH_2 NR_7-,
A-O-$(CH_2CH_2O)_x$-CH_2CH_2 SH,



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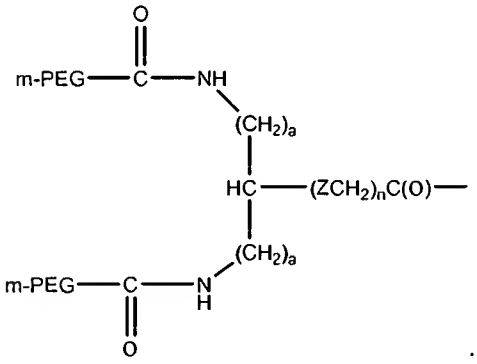


wherein the molecular weight of the polyalkylene oxide polymer ranges from about 20-40 and preferably 30kDa to about 40 kDa.

In order to conjugate the IFN-*beta* to polymers such as poly(alkylene oxides), one of the polymer hydroxyl end-groups is converted into a reactive functional group which allows conjugation. This process is frequently referred to as "activation" and the product

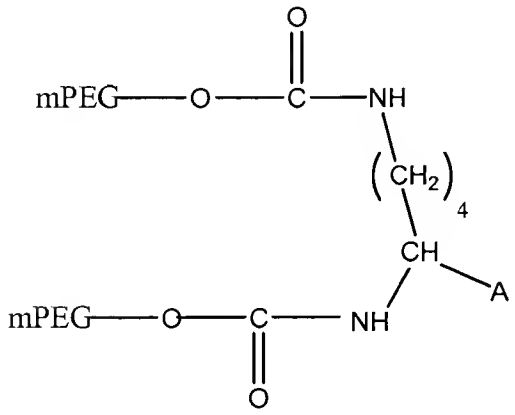
is called an "activated" polymer or activated poly(alkylene oxide). Other substantially non-antigenic polymers are similarly "activated" or functionalized. Polyethylene glycol (PEG) is the most preferred PAO. The general formula for PEG and its derivatives, i.e.

$$A'\text{--}O\text{--}(CH_2CH_2O)_x\text{-}A$$

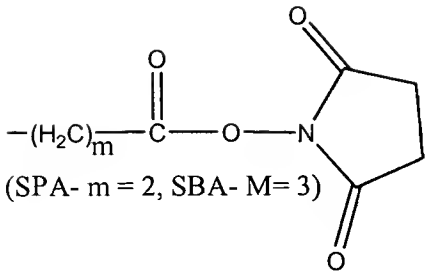
where (x) represents the degree of polymerization or number of repeating units (up to about 2300) in the polymer chain and is dependent on the molecular weight of the polymer. (A) is an activated linking group such as those described below while A' is the same as (A), an alternative activated linking group, H or a capping group such as CH_3. Such mono-activated PEG derivatives are commonly referred to as mPEG derivatives. In addition to mPEG, it should be generally understood that PEGs terminated on one end with any C_{1-4} alkyl group are also useful.

In alternative aspects, the polymer is a poly(propylene glycol) or PPG. Branched PEG derivatives such as those described in commonly-assigned U.S. Pat. Nos. 5,643,575, 5,919,455 and 6,113,906, "star-PEG's", terminally-branched or forked PEG's and multi-armed PEG's such as those described in Nektar catalog "Polyethylene Glycol and Derivatives for Advanced PEGylation 2003". The disclosure of each of the foregoing is incorporated herein by reference. A non-limiting list of PEG derivatives is provided below:

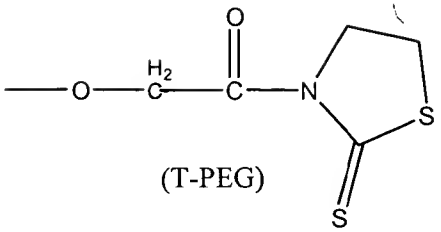
mPEG-A, A-PEG-A and



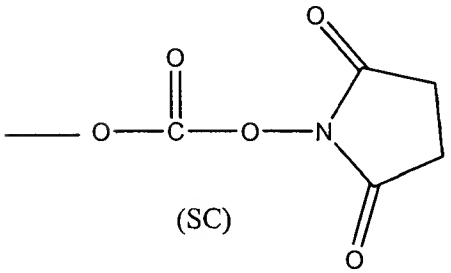
A non-limiting list of suitable PEG activated linking groups is provided below. The activated linking groups correspond to A in the formula given above.



(SPA- m = 2, SBA- M= 3)



(T-PEG)



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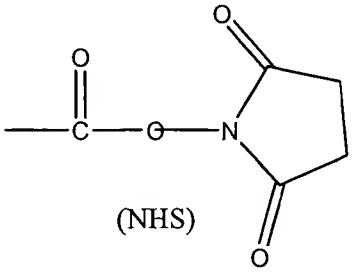


(NHS)

	$CH_2CH_2CH_2$-CHO	-CHO
5	(PEG-Butyraldehyde)	(PEG-aldehyde)

The foregoing can be attached to alpha and/or omega terminal of the PEG, it being understood that when both such linking groups are employed, the resulting conjugates can have two (2) equivalents of IFN-*beta* per unit of polymer.

10 As will be appreciated by those of ordinary skill, the aldehyde derivatives are used for N-terminal attachment of the polymer to the IFN. For example, polyalkylene oxide (PAO) aldehydes react only with amines and undergo reductive amination reactions with primary amines in the presence of sodium cyanoborohydride to form a secondary amine. Suitable polyethylene glycol (PEG) aldehydes are available from 15 Nektar of San Carlos, CA. In other aspects of the invention, the other activated linkers shown above will allow for non-specific linkage of the polymer to Lys amino groups- forming carbamate (urethane) or amide linkages.

In some preferred aspects of the invention when Lys attachment is desired, the activated linker is an oxycarbonyl-oxy-N-dicarboximide group such as a succinimidyl

carbonate group. Alternative activating groups include N-succinimide, N-phthalimide, N-glutarimide, N-tetrahydrophthalimide and N-norborene-2,3-dicarboxide. These urethane-forming groups are described in commonly owned U.S. Pat. No. 5,122,614, the disclosure of which is hereby incorporated by reference. Other urethane-forming activated polymers such as benzotriazole carbonate activated (BTG-activated PEG-available from Nektar) can also be used. See also commonly-assigned U.S. Pat. No. 5,349,001 with regard to the above-mentioned T-PEG.

It will also be appreciated that heterobifunctional polyalkylene oxides are also contemplated for purposes of cross-linking IFN-*beta*, or providing a means for attaching other moieties such as targeting agents for conveniently detecting or localizing the polymer-IFN-*beta* conjugate in a particular areas for assays, research or diagnostic purposes.

In many aspects, suitable polymers will vary somewhat by weight but are preferably at least about 20,000 (number average molecular weight). Alternatively, the polymers may range from about 20,000 to about 60,000, with from about 30,000 to about 40,000 being preferred. In some aspects of the invention where bifunctional PEG is used, the molecular weight can be as low as 20,000.

As an alternative to the preferred PAO-based polymers, other effectively non-antigenic, terminally functionalized polymers such as dextran, polyvinyl alcohols polyvinyl pyrrolidones, polyacrylamides such as HPMA's-hydroxypropylmethacryl-amides, polyvinyl alcohols, carbohydrate-based polymers, copolymers of the foregoing, and the like can be used if the same type of activation is employed as described herein for PAO's such as PEG. Those of ordinary skill in the art will realize that the foregoing list is merely illustrative and that all polymeric materials having the qualities described herein are contemplated. For purposes of the present invention, "effectively non-antigenic" and "substantially non-antigenic" shall be understood to include all polymeric materials understood in the art as being substantially non-toxic and not eliciting an appreciable immune response in mammals.

The activated polymers are reacted with IFN-*beta* under conditions suitable to permit attachment at protein sites that do not significantly interfere with biological

activity, e.g., so that the conjugated IFN-*beta* retains antiviral and other desirable biological activity. Histidine groups, free carboxylic acid groups, suitably activated carbonyl groups, oxidized carbohydrate moieties and mercapto groups, if available on the IFN-*beta* of interest, can also be used as supplemental attachment sites, when appropriate.

In one embodiment, the PEG-IFN-beta-1b conjugate of the composition is present at a concentration of from about 0.01 mg/ml to about 4.0 mg/ml. In other embodiments the protein conjugate is present at a concentration of from about 0.05 mg/ml to about 3.0 mg/ml.

C. Buffers, Surfactants and Excipients

The compositions of the present invention contain a buffer which may be selected from the group consisting of Glycine-HCl, acetic acid, sodium acetate, sodium aspartate, sodium citrate, sodium phosphate and sodium succinate.

Preferably, the buffer is selected from sodium acetate, sodium citrate and glycine HCl. In addition, the buffer preferably has an ionic strength of about 10mM and is present in a concentration of from about 1 mM to about 10 mM. Preferably the buffer is present at a concentration of from about 3 mM to about 5 mM.

The compositions of the present invention also contain an excipient wherein the excipient is non-ionic and is selected from the group consisting of, monosaccharides, disaccharides, and alditols.

Preferably, the excipient is selected from the group consisting of monosaccharides such as, glucose, ribose, galactose, D-mannose, sorbose, fructose, xylulose, and the like, disaccharides such as, sucrose, maltose, lactose, trehalose and the like, polysaccharides such as, raffinose, maltodextrins, dextrans, and the like and alditols such as glycerol, sorbitol, mannitol, xylitol, and the like.

More preferably, the excipient is selected from the group consisting of sucrose, trehalose, mannitol and glycerol or a combination thereof, with the group consisting of mannitol and sucrose or a combination thereof being most preferred.

For the compositions of the present invention, mannitol can be present at a concentration of from between 1 % to about 6 %, sucrose can be present in a

concentration from about 8 % to about 10 % and trehalose can be present in a concentration of from about 8 % to about 10 %. Preferably, the compositions contain about 5 % mannitol or about 9 % sucrose or 9 % trehalose.

The compositions of the present invention further contain a surfactant, wherein the surfactant is non-ionic and is selected from the group consisting of polysorbate 80 (Tween 80), polysorbate 20 (Tween 20), and polyethylene glycol. In one embodiment, the surfactant is polysorbate 80. In one embodiment, the surfactant Tween 80 is present at a concentration of from about 0.01 % to about 0.5 %. Preferably, for compositions of the present invention, Tween 80 is present in a concentration of about 0.5 %.

Reaction Conditions

Details concerning specific reaction conditions which are suitable for making monoPEGylated compounds are provided in the examples. However, the processes of the present invention generally include reacting interferon-*beta* 1b with an activated polyalkylene oxide polymer having a molecular weight of at least about 30 kDa under conditions sufficient to cause conjugation of the activated polyalkylene oxide polymer to the interferon-*beta* 1b, and retaining at least a portion of the antiviral activity relative to native interferon-*beta* 1b, using the standard assay measurements. A non-denaturing surfactant, such as a non-ionic detergent or a zwitterionic detergent, was present as a component in the PEGylation reaction. The preferred surfactant is a zwitterionic detergent. The more preferred is a sulfobetaine, such as Zwittergent 3-14. The reaction conditions for effecting conjugation further include conducting the attachment reaction with from about equi-molar to about a relatively small molar excess of the activated polymer with respect to the IFN. In this regard, the process can be carried out with about 1-15-fold molar excesses; preferably about 2-12-fold molar excesses and most preferably about 3-10-fold molar excesses. The conjugation reaction can be carried out at about room temperature, 20-25° C. It is also preferred that the coupling reaction be allowed to proceed for rather short periods of time, i.e. 0.5-2 hours, before quenching. It was determined that reaction with the aldehyde-activated polymers was best conducted at pH of about 5.2, with later addition of the reducing agent, sodium cyanoborohydride. In

practice, the non-aldehyde-activated polymers result in the formation of a mixture of polymer-IFN positional isomers. Preferably, each isomer contains a single polymer strand attached to the interferon via an amino acid residue. In alternative embodiments, there can be more than one strand of polymer attached to the IFN as a result of the Lys directed processes. Solutions containing these conjugates are also useful as is or can be further processed to separate the conjugates on the basis of molecular weight.

Due to the nature of the solution-based conjugation reactions, the Lys-attached compositions are a heterogeneous mixture of species which contain the polymer strand(s) attached at different sites on the interferon molecule. In any solution containing the conjugates, it is likely that a mixture of at least about 2, preferably about 6 and more preferably about 8 positional isomers will be present.

Methods of Treatment

Another aspect of the present invention provides methods of treatment for various medical conditions in mammals, preferably humans. The methods include administering an effective amount of a pharmaceutical composition that includes an IFN-*beta*-polymer conjugate prepared as described herein, to a mammal in need of such treatment. The conjugates are useful for, among other things, treating interferon-susceptible conditions or conditions which would respond positively or favorably as these terms are known in the medical arts to interferon-based therapy.

Conditions that can be treated in accordance with the present invention are generally those that are susceptible to treatment with IFN-*beta*. For example, susceptible conditions include those which would respond positively or favorably as these terms are known in the medical arts to IFN-*beta*-based therapy. Exemplary conditions which can be treated with IFN-*beta* include, but are not limited to, multiple sclerosis and other autoimmune disorders, cell proliferation disorders, cancer, viral infections and all other medical conditions know to those of ordinary skill to benefit from interferon-*beta* and/or interferon-*beta* 1b therapy. In a preferred aspect of the invention, the polymer conjugated IFN-*beta* is administered to patients in amounts effective to treat multiple sclerosis.

A further aspect of the invention provides for the treatment of conditions that can be treated with polymer-conjugated IFN-*beta*, and preferably polymer-conjugated IFN-*beta* 1b, that have heretofore not fully responded to such treatment because the negative side effects previously outweighed the benefits of the treatment at a given dosage. For example, IFN-*beta* has been tested for treating poor-prognosis Kaposi sarcoma related to HIV/AIDs infection (Miles et al., 1990 Ann Intern Med. 112(8):582-9 and the data suggested a minimal potential benefit. Practice of the invention would allow treatment of this condition, and others, at higher doses and in combination with other art-known therapeutic agents.

Methods of Administration

Administration of the described dosages may be every other day, but is preferably once or twice a week. Doses are usually administered over at least a 24 week period by injection or infusion. Administration of the dose can be intravenous, subcutaneous, intramuscular, or any other acceptable systemic method, including subdermal or transdermal injection via conventional medical syringe and/or via a pressure system. Based on the judgment of the attending clinician, the amount of drug administered and the treatment regimen used will, of course, be dependent on the age, sex and medical history of the patient being treated, the stage or severity of the specific disease condition and the tolerance of the patient to the treatment as evidenced by local toxicity and by systemic side-effects. Dosage amount and frequency may be determined during initial screenings of neutrophil count.

The amount of the IFN-*beta*-polymer conjugate composition administered to treat the conditions described above is based on the IFN activity of the polymeric conjugate. It is an amount that is sufficient to significantly affect a positive clinical response. Although the clinical dose will cause some level of side effects in some patients, the maximal dose for mammals including humans is the highest dose that does not cause unmanageable clinically-important side effects. For purposes of the present invention, such clinically important side effects are those which would require cessation of therapy due to severe flu-like symptoms, central nervous system depression, severe gastrointestinal disorders,

alopecia, severe pruritus or rash. Substantial white and/or red blood cell and/or liver enzyme abnormalities or anemia-like conditions are also dose limiting.

Naturally, the dosages of the various *IFN-beta* conjugate compositions will vary somewhat depending upon the *IFN-beta* moiety and polymer selected. In general, however, the conjugate is administered in amounts ranging from about 100,000 to about 1 to 50 million IU/m^2 per day, based on the condition of the treated mammal or human patient. The range set forth above is illustrative and those skilled in the art will determine the optimal dosing of the conjugate selected based on clinical experience and the treatment indication.

EXAMPLES

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention.

EXAMPLE 1

PRODUCTION OF RECOMBINANT IFN- *beta*

The *E. coli* codon optimized gene for IFN-beta-1b was expressed in the BL21/pET system which employs the T7 RNA polymerase expression control. The IFN-beta-1b protein was expressed in inclusion bodies comprising about 30% of total cell protein. After solubilization and butanol extraction, the protein was purified to near homogeneity by DEAE and SP ion exchange chromatography in the presence of zwittergent 3-14. All other standard recovery steps were employed.

EXAMPLE 2

PREPARATION of ALD-PEG-IFN -*Beta*-1b CONJUGATES

In this example, $mPEG_2$ aldehyde 40,000 (2x20,000) (ALD-PEG) was obtained from Nektar Therapeutics, Huntsville, AL. Next, the IFN-ß of Example 1 was incubated with the ALD-PEG at 1:10 reaction molar ratio in 100 mM sodium acetate, 0.05 % zwittergent, at a pH of about 5.2, at 25 °C for about 3 hours. Sodium cyanoborohydride at 1 M in PEGylation buffer was added to a final concentration of 15 mM. The reaction

was conducted at 25°C for about 16 hours. The mono-ALD-PEG-IFN was purified by a cation exchange column.

EXAMPLES 3-4

Preparation of PEG2-40k-IFN and PEG-UA-40k-IFN

In these Examples, activated PEG2-40k-NHS and PEG2-40k-beta alanine-NHS obtained from Nektar Therapeutics, Huntsville, AL, and Enzon, Inc., respectively, were each separately incubated with the IFN-ß of Example 1. With fast stirring, each amine activated PEG powder was separately added to 0.3-0.8 mg/ml IFN-beta in 50-100 mM sodium phosphate, pH 7.8, 2 mM EDTA, and 0.05% Zwittergent at 0.5-1.0 g/min. The reaction molar ratio of PEG:IFN was about 10:1. After 60-min reaction at 25°C, each reaction was quenched by lowering pH to 6.0 with 2 N HAc. The mono PEG-IFN-beta conjugate in each case was purified on a cation exchange column.

EXAMPLE 5

di PEG-20k-IFN

The same PEGylation conditions employed in Examples 3 and 4 were employed as above except the reaction molar ratio was about 1:20. After 60 min reaction with PEG-20k-SPA, obtained from Nektar Therapeutics, di PEG-20k-IFN was purified by a size exclusion column, followed by a cation exchange column.

EXAMPLE 6

Methods to Detect Aggregation

- The samples were buffer-exchanged to the buffers described in this invention, using Centricon YM-30 (Millipore Corp., Bedford, MA).
- To accelerate the study, the samples were placed at 37°C and under N_2 for 24 hrs. The stability was monitored on SEC-HPLC.
- Size exclusion chromatography HPLC (Superdex 200, HR, Amersham Biosciences, Piscataway, NJ). The buffer system comprises 0.1 M sodium phosphate, pH 6.8.
- RP-HPLC to detect degradation.
- Change of the intrinsic fluorescence wavelength and intensity for the conjugates.
- Non reducing SDS-PAGE.

- Particle size analysis (Dynapro).
- Antiviral and antiproliferation activities
- ELISA

Definition of Aggregation

- A physical aggregation of monomer to form dimer, trimer, tetramer, or multimer which may or may not precipitate in the formulation buffers and the conditions examined.
- The soluble aggregate will be converted to monomer on non reducing SDS gel.
- The soluble aggregate will be reversed to monomer upon dilution.

Liquid Formulation

Example 6A: The lower pH of formulation buffer is preferred

- Organic and inorganic buffers with pH ranging from 3.0 to 11.0 were examined. Glycine-HCl, pH 3.0, acetic acid, pH 3.7, sodium acetate, pH 4.5, sodium succinate, pH 4.4, sodium aspartate, pH 5.4, sodium citrate, pH 3-6, and sodium phosphate, pH 6.0-7.4 were used as basic buffers for examining effects of excipients. In the presence of 3 mM HAc, pH 3.7, the conjugate was stable at 37°C for at least 17 days.
- The preferred buffers are acetate (free acid or salt), citrate (free acid or salt), and glycine-HCl. Citrates have a dual role as chelating agents.
- The preferred pH is acidic, more specifically between pH 3.0 and 4.0.
- The preferred concentrations of the buffers at pH 3.0-4.0 are below 10 mM. Citrate buffers at > 50 mM will result in excess pain on subcutaneous injection and toxic effects due to the chelation of calcium in the blood.

Effect of Buffer pH on Aggregation*

Buffer	Concentration	Excipient	pH	Protein (mg/ml)	T (°C)	Time (day)	Aggregation (%)
Acetic acid	3 mM		3.7	0.1	4	37	0
Citrate	5 mM		4.0				0
Citrate	5 mM		5.0				3.5
Citrate	5 mM		6.0				4.1
Na Phospahte	5 mM		7.4				6.7
Na phosphate	5 mM		8.5				57.3
H_2O							4.7
Acetic acid	3 mM	mannitol, 5%	3.7	0.25	37	11	1.8
						26	6.7

*The percent aggregation was analyzed by SEC-HPLC.

Example 6B. Excipients of Carbohydrates

- Non-ionic tonicity modifying agents were examined as bulking agents to stabilize the conjugate and to render the compositions isotonic with body fluid.
- As classified in textbooks, monosaccharides include glucose, ribose, galactose, D-mannose, sorbose, fructose, xylulose, and the like; disaccharides are sucrose, maltose, lactose, trehalose, and the like, and polysaccharides comprise raffinose, maltodextrins, dextrans, and the like. Alditols contain glycerol, sorbitol, mannitol, xylitol, and the like.
- The preferred non-ionic agents are sucrose, trehalose, mannitol, and glycerol, or a combination thereof. The more preferred non-ionic bulking agents are mannitol and sucrose, or a combination thereof.
- The preferred compositions of the tonicifying agents are 4-6% mannitol, 8-10% sucrose, or 8-10% trehalose. The more preferred compositions are 5% mannitol and 9% sucrose or trehalose.
- The negatively charged polysaccharides such as heparin and chondroitin sulfate at 0.5 mg/ml to 20 mg/ml did not help in preventing the aggregation of the conjugate at neutral pH.

Example 6C. Lower ionic strength is preferred at lower pH while higher ionic strength is preferred at high pH.

- Increasing ionic strength with reagents such as NaCl, KCl, $CaCl_2$ was not favored in keeping the conjugates from aggregation at acidic pH. Their presence at 140 mM, pH 3.7, facilitated aggregation.
- At pH 5.5 to 7.5, the higher ionic strength is preferred over lower ionic strength in preventing the protein from aggregation. For example, 100 mM sodium phosphate, at pH 7.4 is better than its 10 mM concentration in preventing the aggregation.
- The ionic strength of a solution is expressed as ½ the sum of $CiZi^2$ where C is the concentration, Z is the charge, and i represents ion.
- Low ionic strength is preferred in low pH buffers while high ionic strength is preferred in high pH buffers. The preferred ionic strength in pH 3.0-4.0 buffers is lower than 10 mM and the preferred ionic strength in pH 5.5-7.5 buffers is 100-150 mM.

Example 6D. Effect of Surfactants

- Non-ionic surfactants include polyoxyethylene sorbitol esters such as polysorbate 80 (Tween 80) and polysorbate 20 (Tween 20) and polyethylene glycol.
- Zwitterionic surfactant such as zwittergent was used to solubilize unmodified protein.
- The preferred non-ionic surfactants are polysorbate 80, polysorbate 20, and polyethylene glycol.
- Polysorbate 20 from Sigma prevented protein aggregation whereas Polysorbate 20 from Calbiochem and J. T. Baker did not.

Example 6E. Low storage temperature

- Stability of the protein at different temperatures in various buffers, pHs, and excipients was investigated.
- The stability of the protein decreases with elevated temperatures: -20°C, 4°C > 25°C > 37°C. The temperature of 37°C was used to accelerate the stability study. The preferred temperatures are -20°C and 2-8°C.
- At 2-8°C, the conjugates were stable even in the presence of unfavorable components such as high salt (140 mM NaCl) or high pH (pH 7.4) for at least a few weeks.
- In low pH (3.0- 4.0) and low ionic strength buffers, ten cycles of freeze-thaw from -80 °C to +20 °C caused about 2% aggregation. Each cycle of freeze-thaw from -80 °C to +37 °C caused about 3% aggregation.)
- In pH 5.0-6.5 buffers, for example, 10 mM sodium acetate, pH 5.0, 150 mM NaCl, or 10 mM sodium phosphate, pH 6.5, 150 mM NaCl, in the presence or absence of Polysorbate 80, five cycles of freeze-thaw from -80 °C or -20 °C to +20 °C did not cause any aggregation or a loss of antiviral activity.

Example 6F. Protein concentration

- PEG-IFN-β-1b concentrations between 0.125-4 mg/ml were examined. The samples were incubated in 5% mannitol, 3 mM HAc (pH 3.7), 37°C and under N_2 for 24 hours. The integrity of the conjugate was monitored by SEC-HPLC.
- Lowering protein concentration lowered protein aggregation.
- The preferred protein concentrations are between 3.0 mg/ml to 0.05 mg/ml.

Effect of Protein Concentration

Batch #	Buffer	Time (day)	Concentration (mg)	Aggregation (%)
30814M40	3 mM acetic acid, pH 3.7	17 at 37°C	0.05 0.10	3.3 2.0
31212M40	5% mannitol, 3 mM acetic acid, pH 3.7	1 at 37°C	0.125 0.25 0.5 1.0 2.0 4.0	0 0 0.3 0 0 5.7

Example 6G. Neutralization of solution pH for administration

- Acidic solution could cause skin irritation.
- Acidity can be neutralized by adding sodium phosphate solution or powder before administration. For example, when 1/10 volume of 10xPBS or powder with the same components was added to 1% mannitol, 3 mM HAc, pH 3.7, 0.30 mg/ml PEG-protein, the pH increased to 6.5.

- The sample after neutralization should be administered within 2 hrs at 25°C or 20 hrs at 4°C.

Effect of Neutralization on Aggregation*

Neutralization	pH	T (°C)	Time after neutralization (hr)	Aggregation (%)
no	3.7	25	0	0
yes	6.5	25	0	0.5
			0.5	0.1
			1.0	2.7
			2.0	3.0
			18	13.8
		4	0	0
			19	2.2

*Percent aggregation was analyzed by SEC HPLC

EXAMPLE 7
Lyophilization

Example 7a: Addition of Mannitol in lyophilization Buffer.

- Inclusion of mannitol in lyophilization buffer reduced aggregation and retained antiviral activity after reconstitution.
- The ratio of PEG2-40k-IFN-β-1b to mannitol was 0.5-2.5% by weight.
- 1% mannitol was preferred.

Addition of Mannitol in Lyophilization Buffers*

	Mannitol (%)	Monomer (%)		Antiviral activity (MU/mg	
		control, no lyophilization	lyophilization	control, no lyophilization	lyophilization
IFN-β-1b	0	N/A	N/A	16.97	4.71
	0.1	N/A	N/A	15.36	12.03
	0.2	N/A	N/A	12.66	9.44
	1	N/A	N/A	10.28	10.39
PEG2-40k-IFN-β-1b	0	92	91	6.07	4.2
	0.1	92	92	3.15	4.85
	0.2	92	92	4.21	4.23
	1	93	92	5.47	4.27

*The protein concentration was 0.3-0.4 mg/ml and lyophilization buffers contained 3 mM HAc, pH 3.7 and mannitol as indicated. The reconstitution buffer was 3 mM HAc, pH 3.7.

Example 7b: Addition of Polysorbate to Lyophilization Buffers

- Addition of polysorbate 80 in lyophilization buffers retained antiviral activity of PEG-protein after reconstitution.

Addition of Polysorbate 80 in Lyophilization Buffer*

Polysorbate 80 (%)	Antiviral Activity (MU/mg)**	
	control, no lyophilization	lyophilization
0	5.18	3.79
0.02	4.35	2.54
0.1	4.61	3.56
0.5	5.85	5.18

*The lyophilization buffer contained 5% mannitol, 3 mM HAc, and polysorbate 80 at the concentration indicated. The reconstitution buffer was 10 mM sodium phosphate, pH 7.4. **Vero cell assay.

Example 7c: Effect of Reconstitution Buffer on PEG-protein Aggregation

- Three reconstitution buffers at pH 7.4 (10 mM sodium phosphate), pH 5.0 (10 mM sodium acetate), and 3.7 (3 mM acetic acid) were compared for the amount of aggregation of PEG-protein after lyophilization and reconstitution.
- The lower the pH of the reconstitution buffers, the lower the amount of the aggregation.
- The preferred lyophilization buffer contained 0.1-2 mg/ml PEG-protein, 0.1-5% mannitol, 3 mM HAc, pH 3.7, and 0.02-0.5% polysorbate 80 from J. T. Baker.
- The powder after lyophilization was reconstituted in 10 mM sodium acetate or sodium phosphate, pH 5.0-7.4, 0-140 mM NaCl.
- Alternatively, the reconstitution buffer was 3 mM HAc, pH 3.7 to make 0.1-2 mg/ml PEG-protein. 10 mM Sodium phosphate, pH 7.4 was added to neutralize the pH before administration.

Effect of Reconstitution Buffer*

Buffer	pH	T (°C)	Time after reconstitution (hr)	Aggregation (%)
3 mM Hac	3.7	25	0 6	2.3 1.8
3 mM HAc, then neutralized with 10 mM sodium phosphate, pH 7.4	6.5	25	0 2.5 4.5	2.2 3.4 4.1
10 mM sodium phosphate, pH 6.5, 120 mM NaCl	6.5	25	0 7	4.3 5.4

*Percent aggregation was analyzed by SEC HPLC

CONCLUSIONS

From the foregoing the following preferred aspects of the invention are shown:

1. The preferred buffers (at -20 °C, -80 °C, or +4 °C) which are composed of glycine-HCl, citrate, acetate, or aspartate with pH between 3.0 and 5.0 and the concentration between 5-10 mM.
2. The ionic strength in the buffers in (1) is lower than 10 mM.
3. The buffers which are composed of glycine-HCl, citrate, acetate, aspartate, phosphate, and carbonate with pH 3-8.
4. Excipients of carbohydrates are mannitol, sorbitol, sucrose, trehalose, and glycerol, or a combination thereof.
5. Excipients of surfactants are polysorbate 80, polysorbate 20, polyethylene glycol.
6. Neutralization of acidic buffers with sodium phosphate before administration.
7. Inclusion of mannitol, sucrose, or trehalose, or a combination thereof at 0.1-5% (w/v) in lyophilization buffer.
8. Inclusion of polysorbate 80 or polysorbate 20 at 0.002-0.5% (w/v) in lyophilization buffers in (7).
9. The reconstitution buffer was sodium acetate or sodium phosphate, pH 5.0-7.4, plus NaCl to isotonicity.
10. The reconstitution buffers were glycine-HCl, citrate, acetate, or aspartate with pH 3.0-4.0, followed by neutralization with sodium acetate or sodium phosphate to final pH 5.0-7.4 for administration.
11. Formulation of the PEGylated recombinant proteins in the buffers claimed described above.
12. The inactive pharmaceutical ingredients after reconstitution of the lyophilized PEGylated proteins are those described above.

EXAMPLE 8.

Immunogenicity and *In Vitro* Stability

- Experimental design: Sprague Dawley (Harlan) rats weighing 150-300 g (three in a group) were administered intramuscularly or subcutaneously with native IFN-β-1b or PEG-IFN-β-1b conjugates at 0.1 mg/kg, once per week for 3-6 weeks. The plasma samples were collected seven days after the previous injection and right before the next injection.
- Assay design: the antibodies produced against IFN-β-1b or PEG-β-1b conjugates were analyzed by direct ELISA where the capture reagent was IFN-β-1b and detection antibody was horse radish peroxidase conjugated rabbit against rat IgG.

Analysis of Rat Anti hIFN-β-1b Antibodies by ELISA (μg/ml)*

Antigen	QWx1	QWx2	QWx3	QWx4	QWx6
IFN-β-1b	4.89±4.42	119.8±84.77	161.87±97.82	305.37±28.88	233.16±55.75
ALD-PEG-40k	6.47±0.35	3.46±1.82	9.70±8.95	13.03±6.56	7.20±2.62
PEG2-40k	3.52±2.74	7.29±1.66	7.93±0.03	12.59±0.66	7.53±1.18
PEG-U-Ala-40k	7.49±1.73	3.65±3.26	4.42±3.56	8.03±5.20	5.02±1.93
Di PEG-20k	6.07±2.42	4.98±4.47	3.54±0.13	8.44±5.91	4.40±2.28

* Mouse anti h IFN-β monoclonal antibody (R&D, #21405-1, clone#MMHB-3, IgG1, κ) was used as standard.

See also Figure 1

CONCLUSIONS

From the foregoing the following preferred aspects of the invention are shown:

- PEGylation greatly reduced immunogenicity of the protein.
- Immunogenicity (IgG titers) of IFN-β-1b was reduced by 94-98% after PEGylation with mono PEG-40k and di PEG-20k.
- Rat immune system was more tolerant of PEG-protein than the native protein. There was no significant increase of antibodies from first to sixth dose.
- The antibodies were neutralizing antibodies when analyzed by antiviral bioassays.
- There was no increased production of antibodies after 4 doses with i.m. administration.
- We further discovered that the PEG-IFN-beta compounds were more resistant toward proteases in mouse kidney and liver extracts upon PEGylation. The half life of IFN-β-1b increased by 6 fold in both kidney and liver extracts after PEGylation. The stability was analyzed by ELISA.
- We further discovered that the PEG-IFN-beta compounds were more resistant toward oxidation by hydrogen peroxide after PEGylation.

EXAMPLE 9.
Enhanced Pharmacokinetic Profiles

Pharmacokinetic Parameters in Rats

Compound	Route	Dose (mg/kg)	Half-life (hr)	AUC (hr.u/mL)	Clearance (ug/hr/(unit/ml))	Tmax (hr)	Cmax (hr)
IFN-β-1b	iv	0.6	1.08	26210	0.0057	NA	
PEG2-40k	iv	0.6	9.43	751328	0.0002	NA	
PEG-U-Ala-40k	iv	0.6	12.0	687389	0.0002	NA	
IFN-β-1b	sc	0.6	2.43	323.9	0.399	1.0	95.3
PEG2-40k	sc	0.6	23.8	72014	0.0019	24	1829.2
PEG-U-Ala-40k	sc	0.6	18.1	42938	0.0032	48	798.3
IFN-β-1b	im	0.6	2.29	805.1	0.15	0.5	135.8
PEG2-40k	im	0.6	15.2	164920	0.0009	8.0	4908.6
PEG-U-Ala-40k	im	0.6	14.4	76782	0.0019	8.0	2989.8

*By Vero cell assay

See also Figure 2

CONCLUSIONS

From the foregoing the following preferred aspects of the invention are shown:

- The AUC of IFN-β-1b was enhanced by more than 90 fold by s.c. or i.m. administration and clearance rate was prolonged by more than 80 fold after mono PEGylation with PEG-40k.
- Bioavailability of PEGylated IFN-β-1b was better by i.m. than by s.c. in both mice and rats.
- Bioavailability of the PEGylated IFN-β-1b was better than the native IFN-beta.

Bioavailability of IFN-β-1b and PEG-IFN-ß-1b Conjugate in Mice and Rats

Compound	Species	Dose (mg/kg)		Bioavailability (%)*	
		s.c.	i.m.	s.c.	i.m.
IFN-β-1b	mouse	0.2	0.1	15	30
PEG2-40k-IFN-β-1b		0.2	0.1	22	42
IFN-β-1b	rat	0.6	0.6	0.96	2.0
PEG2-40k-IFN-β-1b		0.6	0.6	8.9	34

*Average numbers from Vero and A549 antiviral (EMC) cell assays.

WE CLAIM:

1. A composition comprising:

a) interferon conjugated to a polyalkylene oxide polymer having a molecular weight of at least about 20 kDa; and optionally

b) a surfactant;

c) an excipient, and

d) a buffer

wherein the pH range of the solution is from about 3 to about 11.

2. The composition of claim 1 wherein the interferon is interferon-*beta* 1b.

3. The composition of claim 1 wherein the surfactant is selected from the group consisting of polyoxyethylene sorbitol esters and polyethylene glycol.

4. The composition of claim 1 wherein the pH range is from about 2.5 to about 8.5.

5. The composition of claim 1 wherein the pH range is from about 3.0 to about 5.0.

6. The composition of claim 1 wherein the pH range is from about 3.0 to about 4.0.

7. The composition of claim 1 wherein the buffer is selected from the group consisting of Glycine-HCl, acetic acid, sodium acetate, sodium aspartate, sodium citrate, sodium phosphate and sodium succinate.

8. The composition of claim 1 wherein the buffer is selected from sodium acetate, sodium citrate and glycine HCl.

9. The composition of claim 1 wherein the buffer has an ionic strength of about 10mM.

10. The composition of claim 1 wherein the buffer is present in a concentration of from about 3 mM to about 10 mM.

11. The composition of claim 1 wherein the excipient is non-ionic and is selected from the group consisting of, monosaccharides, disaccharides, and alditols.

12. The composition of claim 7 wherein the excipient is selected from the group consisting of glucose, ribose, galactose, D-mannose, sorbose, fructose, xylulose, sucrose, maltose, lactose, trehalose, raffinose, maltodextrins, dextrans, glycerol, sorbitol, mannitol, and xylitol.

13. The composition of claim 8 wherein the excipient is selected from the group consisting of sucrose, trehalose, mannitol and glycerol or a combination thereof.

14. The composition of claim 9 wherein the excipient is selected from the group consisting of mannitol and sucrose or a combination thereof.

15. The composition of claim 1 wherein the surfactant is non-ionic and is selected from the group consisting of polysorbate 80, polysorbate 20, and polyethylene glycol.

16. The composition of claim 1 wherein the polyalkylene oxide polymer is linear or branched.

17. The composition of claim 1 wherein the linear polyalkylene oxide polymer is of the formula:

$$A\text{- }O\text{-}(CH_2CH_2O)_x\text{-}$$
$$A\text{-}O\text{-}(CH_2CH_2O)_x\text{-}CH_2C(O)\text{-}O\text{-},$$
$$A\text{-}O\text{-}(CH_2CH_2O)_x\text{-}CH_2CH_2\ NR_7\text{-},$$
$$A\text{-}O\text{-}(CH_2CH_2O)_x\text{-}CH_2CH_2\ SH,$$
$$\text{-}O\text{-}C(O)CH_2\text{-}O\text{-}(CH_2CH_2O)_x\text{-}CH_2C(O)\text{-}O\text{-},$$
$$\text{-}NR_7CH_2CH_2\text{-}O\text{-}(CH_2CH_2O)_x\text{-}CH_2CH_2\ NR_7\text{-},$$
$$\text{-}SHCH_2CH_2\text{-}O\text{-}(CH_2CH_2O)_x\text{-}CH_2CH_2\ SH\text{-},$$

wherein

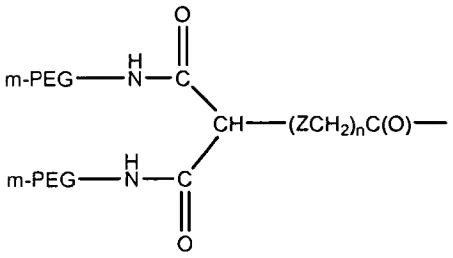
A is a capping group;

R_7 is selected from hydrogen, $C_{1\text{-}6}$ alkyls, $C_{3\text{-}12}$ branched alkyls, $C_{3\text{-}8}$ cycloalkyls, $C_{1\text{-}6}$ substituted alkyls, $C_{3\text{-}8}$ substituted cycloalkyls, aryls, substituted aryls, aralkyls, $C_{1\text{-}6}$ alkenyls, $C_{3\text{-}12}$ branched alkenyls, $C_{1\text{-}6}$ alkynyls, $C_{3\text{-}12}$ branched alkynyls, $C_{1\text{-}6}$ heteroalkyls, substituted $C_{1\text{-}6}$ hetero-alkyls, $C_{1\text{-}6}$ alkoxyalkyl, phenoxyalkyl and $C_{1\text{-}6}$ heteroalkoxys, and

x is the degree of polymerization.

18. The composition of claim 5 where in said capping group is selected from the group consisting of OH, CO_2H, NH_2, SH, and C_{1-6} alkyl moieties.

19. The composition of claim 1 wherein the branched polyalkylene oxide polymer is selected from the group consisting of:



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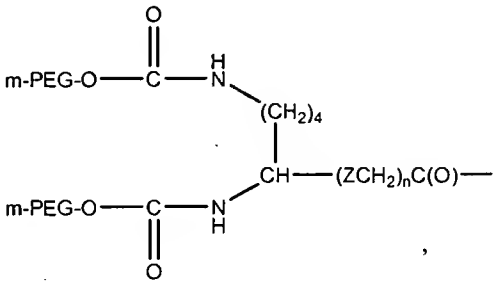

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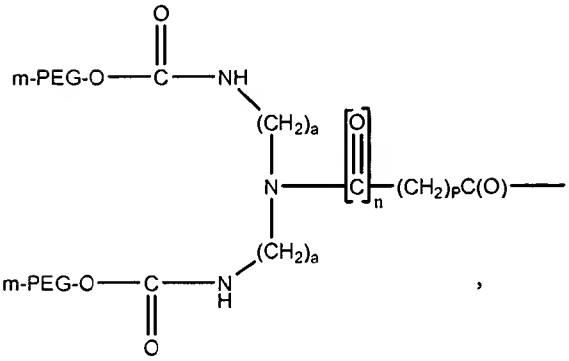

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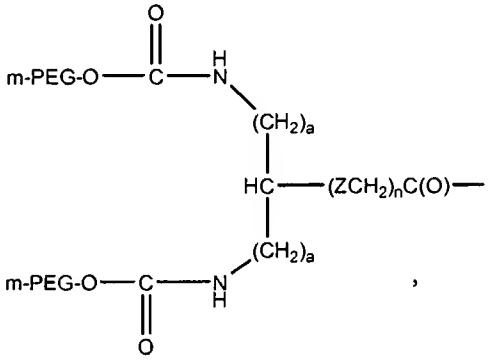

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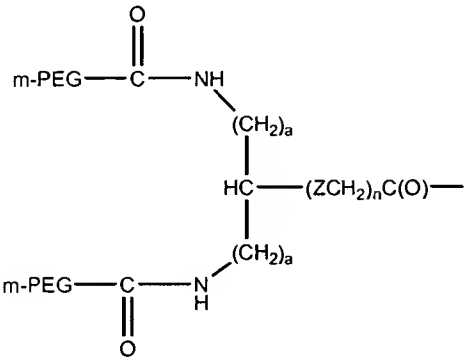


,

wherein:

(a) is an integer of from about 1 to about 5;

Z is O, NR_8, S, SO or SO_2, where R_8 is H, C_{1-8} alkyl, C_{1-8} branched alkyl, C_{1-8} substituted alkyl, aryl or aralkyl;

(x) is the degree of polymerization;

(n) is 0 or 1;

(p) is a positive integer, preferably from about 1 to about 6;

m-PEG is CH3-O-$(CH2CH2O)_x$- , and

The ligand is interferon-beta 1b.

20. The composition of claim 1 wherein the interferon-*beta* 1b comprises the amino acid sequence of SEQ ID NO:1. [*see end of Claims section*]

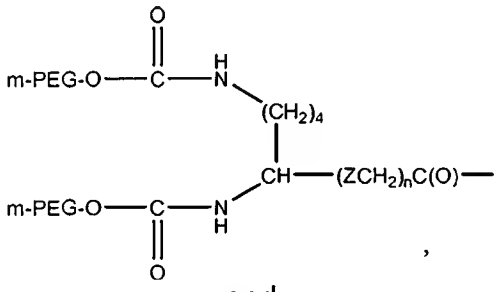
21. The composition of claim 20 wherein the interferon –beta 1b is conjugated to a polyalkylene oxide polymer selected from the group selected from:

A- O-$(CH_2CH_2O)_x$-

A-O-$(CH_2CH_2O)_x$-$CH_2C(O)$-O-,

A-O-$(CH_2CH_2O)_x$-CH_2CH_2 NR_7-,

A-O-$(CH_2CH_2O)_x$-CH_2CH_2 SH,



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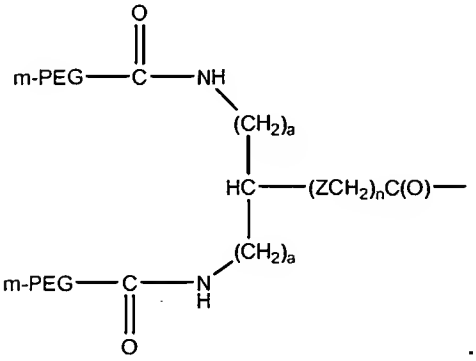


22. The composition of claim 21 wherein the molecular weight of the polyalkylene oxide polymer ranges from about 30kDa to about 60 kDa.

23. The composition of claim 21 wherein the molecular weight of the polyalkylene oxide polymer is about 30 kDa.

24. The composition of claim 21 wherein the molecular weight of the polyalkylene oxide polymer is about 40 kDa.

25. The composition of claim 1 wherein the polyalkylene oxide polymer is conjugated to the interferon-*beta* 1b by a linkage selected from the group consisting of urethane, secondary amine, amide, or thioether.

26. The composition of claim 1 wherein the interferon-*beta* 1b is conjugated to a polyalkylene oxide polymer via the alpha-amino-terminal of the interferon-*beta* 1b.

27. The composition of claim 1 wherein the interferon-*beta* 1b is conjugated to a polyalkylene oxide polymer via an epsilon amino group of a Lys of the interferon-*beta* 1b.

28. The composition of claim 1 wherein the interferon conjugate is present at a concentration of from about 0.01 mg/ml to about 4 mg/ml.

29. The composition of claim 28 wherein the interferon conjugate is present at a concentration of from about 0.05 mg/ml to about 3 mg/ml.

30. A composition comprising:

a) 0.05 to 3.0 mg/ml of interferon beta 1b conjugated to a polyalkylene oxide polymer having a molecular weight of at least about 30 kDa,

b) 1% - 5% mannitol, and

c) 3- 10 mM acetic acid

wherein the pH is about 3.7.

31. A biologically-active polymer-interferon conjugate composition of claim 1, wherein at least about 65 percent of the antiviral activity is retained relative to native interferon-*beta* 1b, using the EMC/Vero or EMC/A549 antiviral bioassay.

32. A biologically-active polymer-interferon conjugate composition of claim 1, wherein at least about 20 percent of the antiviral activity is retained relative to native interferon-*beta* 1b, using the EMC/Vero or EMC/A549 antiviral bioassay.

33. A method of preparing the biologically active polymer-interferon conjugate composition of claim 1, comprising reacting interferon-*beta* 1b with an activated polyalkylene oxide polymer having a molecular weight of at least about 30 kDa under conditions sufficient to cause conjugation of the activated polyalkylene oxide polymer to the interferon-*beta* 1b, purifying the resulting conjugate and resuspending the conjugate in a buffered solution having a pH range of about 3.0 to about 8.0, wherein said solution optionally contains an excipient and a surfactant and wherein said composition retains at least about 20% of the antiviral activity is retained relative to native interferon-*beta* 1b, using the EMC/Vero or EMC/A549 antiviral bioassay.

34. The method of claim 33 wherein the conditions are sufficient to cause conjugation of the activated polyalkylene oxide polymer to the amino-terminal of the interferon-*beta* 1b.

35. The method of claim 33 wherein the conditions are sufficient to cause conjugation of the activated polyalkylene oxide polymer to an epsilon amino group of a Lys of the interferon-*beta* 1b.

36. The method of claim 33 wherein the molecular weight of the activated polyalkylene oxide polymer ranges from about 30kDa to about 40 kDa.

37. The method of claim 33 wherein the molecular weight of the activated polyalkylene oxide polymer is about 30 kDa.

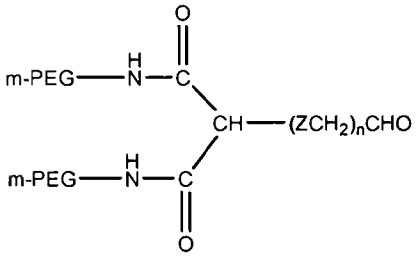
38. The method of claim 33 wherein the molecular weight of the activated polyalkylene polymer is about 40 kDa.

39. The method of claim 33 wherein the activated polyalkylene polymer is an activated polyethylene glycol.

40. The method of claim 39 wherein the activated polyethylene glycol comprises a terminal reactive aldehyde moiety.

41. The method of claim 40 wherein the activated polyethylene glycol is selected from the group consisting of mPEG-$CH_2CH_2CH_2CHO$, mPEG$_2$$CH_2CH_2CH_2CHO$, mPEG-$CH_2CH_2CH_2CH_2CHO$ and mPEG$_2$-$CH_2CH_2CH_2CH_2CHO$.

42. The method of claim 39 wherein the activated polyethylene glycol is selected from the group consisting of



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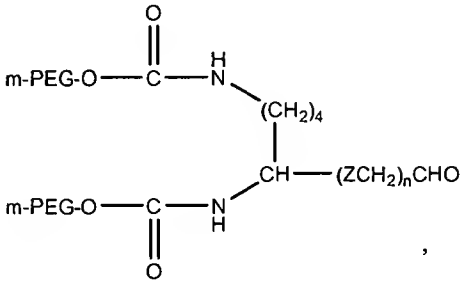

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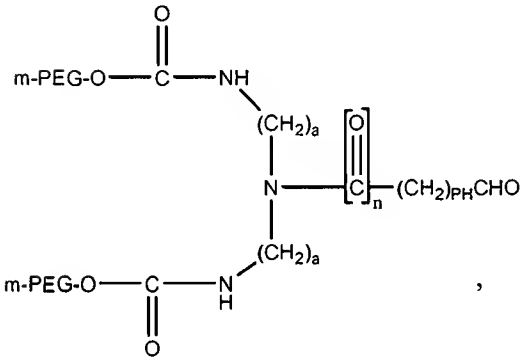

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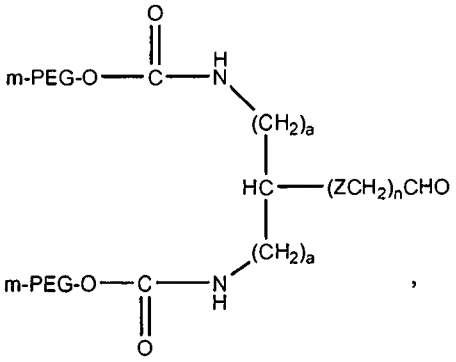

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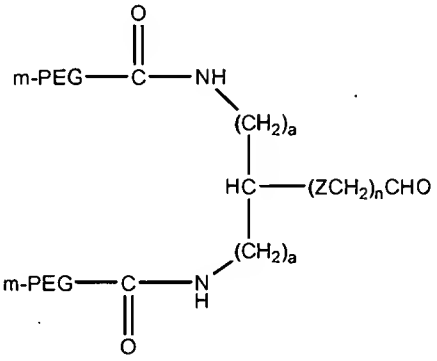


,

wherein:

(a) is an integer of from about 1 to about 5;

Z is O, NR_8, S, SO or SO_2; where R_8 is H, C_{1-8} alkyl, C_{1-8} branched alkyl, C_{1-8} substituted alkyl, aryl or aralkyl;

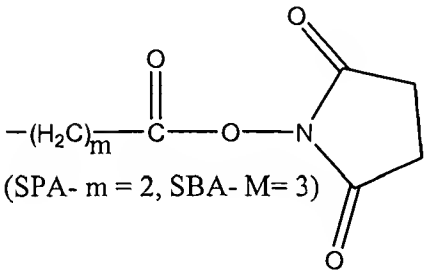
(x) is the degree of polymerization;

(n) is 0 or 1;

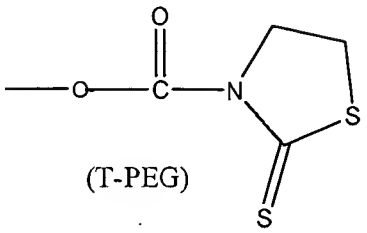
(p) is a positive integer, preferably from about 1 to about 6, and

m-PEG is CH3-O-$(CH2CH2O)_x$- .

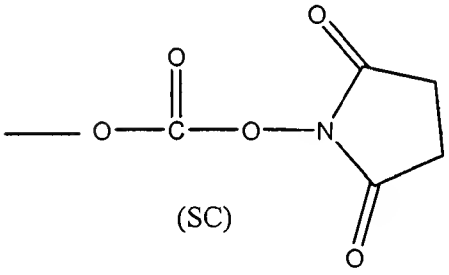
43. The method of claim 33, wherein the activated polyethylene glycol comprises a terminal reactive moiety selected from the group consisting of:



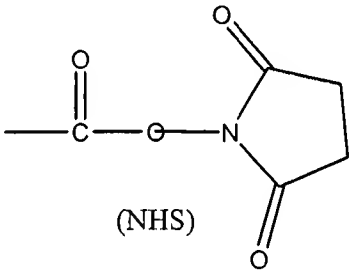
(SPA- m = 2, SBA- M= 3)



(T-PEG)



(SC)



(NHS)

44. A method of administering a composition of claim 1 comprising a first step of neutralizing the acidic buffers followed by adminiistering the composition to a patient in need of such administration.

45 The method of claim 44 wherein the acidic buffer is neutralized with sodium phosphate.

46. The method of claim 44 wherein the composition is administered orally, intravenously, subcutaneously, or intramuscularly.

47. A method of treating a mammal having a disease or disorder responsive to interferon-*beta* comprising administering an amount of the pharmaceutical composition of claim 1 effective to treat the disease or disorder.

SEQ ID NO:1

```
Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln
 2                  5                  10                  15
Ser Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cys Leu
            20                  25                  30
Lys Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys Gln Leu Gln
        35                  40                  45
Gln Phe Gln Lys Glu Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln
    50                  55                  60
Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn
65                  70                  75                  80
Glu Thr Ile Val Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ile Asn
                85                  90                  95
His Leu Lys Thr Val Leu Glu Glu Lys Leu Glu Lys Glu Asp Phe Thr
            100                 105                 110
Arg Gly Lys Leu Met Ser Ser Leu His Leu Lys Arg Tyr Tyr Gly Arg
        115                 120                 125
Ile Leu His Tyr Leu Lys Ala Lys Glu Tyr Ser His Cys Ala Trp Thr
    130                 135                 140
Ile Val Arg Val Glu Ile Leu Arg Asn Phe Tyr Phe Ile Asn Arg Leu
145                 150                 155                 160
Thr Gly Tyr Leu Arg Asn
                165
```

ABSTRACT

Biologically-active, interferon-*beta* 1b-polymer conjugate compositions are disclosed. The polymer portion is preferably a polyalkylene oxide polymer having a molecular weight of at least about 30 kDa. Methods of making and using the same are also disclosed.

Fig. 1

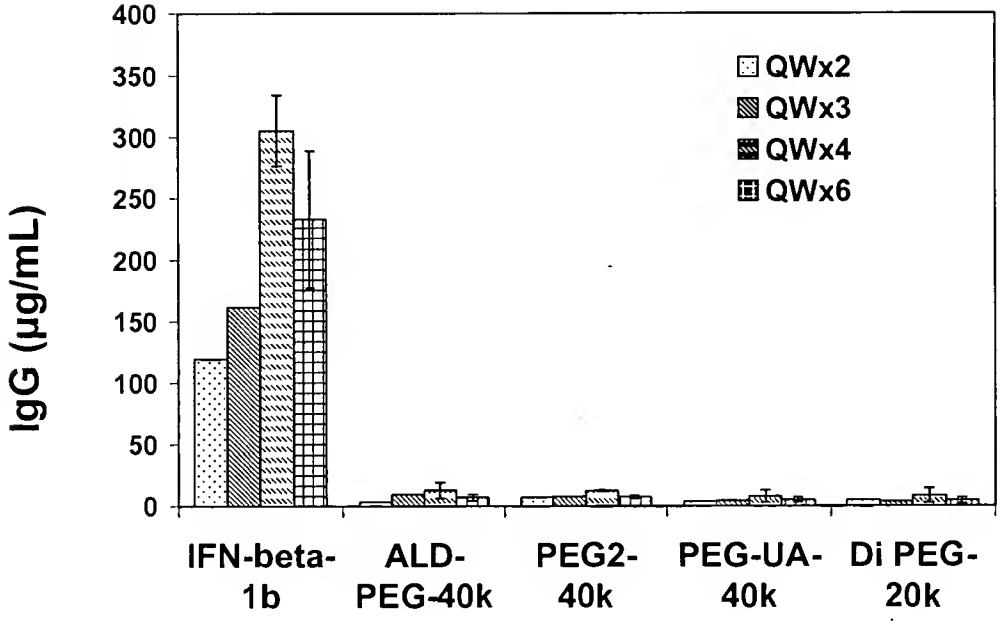
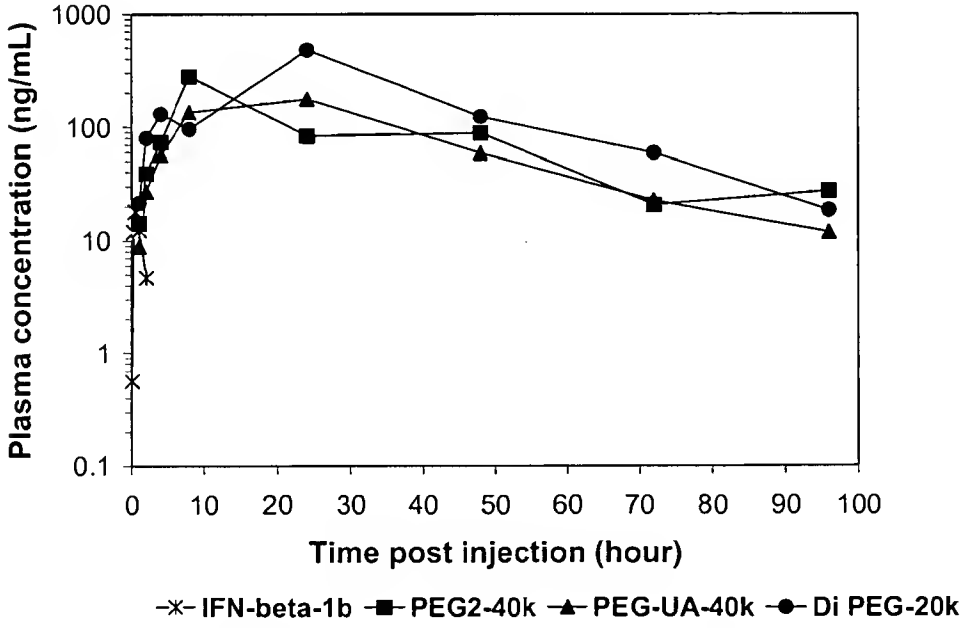

IgG (µg/mL)
400
350
300
250
200
150
100
50
0
QWx2
QWx3
QWx4
QWx6
IFN-beta-1b
ALD-PEG-40k
PEG2-40k
PEG-UA-40k
Di PEG-20k

FIG. 2


Plasma concentration (ng/mL)
1000
100
10
1
0.1
0
10
20
30
40
50
60
70
80
90
100
Time post injection (hour)
IFN-beta-1b
PEG2-40k
PEG-UA-40k
Di PEG-20k